S-nitrosothiol depletion in amyotrophic lateral sclerosis

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Recent data suggest that either excessive or deficient levels of protein S-nitrosylation may contribute to disease. Disruption of *S*-nitrosothiol (SNO) homeostasis may result not only from altered nitric oxide (NO) synthase activity but also from alterations in the activity of denitrosylases that remove NO groups. A subset of patients with familial amyotrophic lateral sclerosis (ALS) have mutations in superoxide dismutase 1 (SOD1) that increase the denitrosylase activity of SOD1. Here, we show that the increased denitrosylase activity of SOD1 mutants leads to an aberrant decrease in intracellular protein and peptide S-nitrosylation in cell and animal models of ALS. Deficient S-nitrosylation is particularly prominent in the mitochondria of cells expressing SOD1 mutants. Our results suggest that SNO depletion disrupts the function and/or subcellular localization of proteins that are regulated by S-nitrosylation such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and thereby contributes to ALS pathogenesis. Repletion of intracellular SNO levels with SNO donor compounds rescues cells from mutant SOD1-induced death. These results suggest that aberrant depletion of intracellular SNOs contributes to motor neuron death in ALS, and raises the possibility that deficient S-nitrosylation is a general mechanism of disease pathogenesis. SNO donor compounds may provide new therapeutic options for diseases such as ALS that are associated with deficient S-nitrosylation.

ALS | GAPDH | S-nitrosylation | mitochondria | superoxide dismutase

Nitric oxide (NO) regulates a diverse array of biological processes by modifying critical cysteine residues on proteins (S-nitrosylation). Proteins acquire NO groups from a pool of intracellular S-nitrosylated peptides. The importance of S-nitrosylation in physiology raises the possibility that dysregulated S-nitrosylation contributes to pathophysiology. Indeed, recent data suggest that excessive NO production and protein S-nitrosylation may contribute to disease. For instance, the E3 ligase parkin is aberrantly S-nitrosylated due to increased NO production in Parkinson's disease (1, 2). S-nitrosylation inhibits parkin activity, leading to the accumulation of misfolded proteins that may contribute to the death of dopaminergic neurons. Similarly, increased NO production during cerebral ischemia increases the S-nitrosylation of matrix metalloproteinase-9 (MMP-9) (3). S-nitrosylation of MMP-9 leads to the formation of a stable sulfinic or sulfonic acid derivative that is irreversibly activated, triggering neuronal apoptosis.

Deficient S-nitrosylation has also been linked to disease pathogenesis. Specifically, increased activity of the denitrosylase *S*-nitrosoglutathione (GSNO) reductase in asthmatic lungs depletes levels of the endogenous bronchodilator GSNO, leading to airway hyperresponsivity (4). Likewise, in sickle cell anemia, the sickle cell hemoglobin is deficient in the intramolecular and intermolecular transfer of NO moieties. Consequently, red blood cell membrane *S*-nitrosothiol (SNO) levels are decreased and hypoxic vasodilation is impaired, leading to vasoocclusion in hypoxic tissue (5).

Here, we investigated whether deficient protein S-nitrosylation contributes to the development of amyotrophic lateral sclerosis (ALS). ALS is one of the most common adult onset neurodegenerative diseases and is characterized by degeneration of motor neurons in the spinal cord, brainstem, and cortex (6). Although the majority of cases are sporadic, ≈10% of cases are familial (FALS), and 20% of the familial cases are due to a mutation in the superoxide dismutase 1 (SOD1) gene (7, 8). SOD1 is an abundant copper- and zinc-containing intracellular protein that converts superoxide to hydrogen peroxide and O_2. Over 90 FALS-associated SOD1 mutations spanning all exons of the *SOD1* gene have been described (8). SOD1 mutants cause motor neuron degeneration by means of a toxic gain-of-function that is not well understood.

WT SOD1 catalyzes the reductive decomposition of S-nitrosylated peptides via a copper-mediated mechanism to yield NO and the corresponding disulfide (9–11). Some ALS-associated SOD1 mutations cause protein misfolding that increases the exposure of the active site copper (12). We hypothesized that increased exposure of the active site copper in SOD1 mutants leads to increased denitrosylase activity. In support of this hypothesis, we have previously shown that two SOD1 mutants (A4V and G37R) catabolize S-nitrosylated peptides significantly faster than WT SOD1 in a cell-free system (13). Because proteins acquire NO groups from S-nitrosylated peptides, a decrease in peptide S-nitrosylation is likely to cause an aberrant decrease in protein S-nitrosylation. Therefore, we further hypothesized that increased denitrosylase activity is a toxic gain-of-function of SOD1 mutants that contributes to motor neuron death in ALS by depleting intracellular SNOs and disrupting the function of proteins that are regulated by S-nitrosylation. To test this hypothesis, in the following studies, we determined whether S-nitrosylated peptide and protein levels are aberrantly decreased in cells and transgenic mice expressing mutant as compared with WT SOD1. We also determined whether repletion of intracellular SNOs with SNO donor compounds improves the survival of cells expressing SOD1 mutants.

Results

GSNO Levels Are Decreased in Cells Expressing SOD1 Mutants. We have previously shown in cell-free systems that ALS-associated SOD1 mutants have increased denitrosylase activity as compared

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Abbreviations: SNO, *S*-nitrosothiol; SOD, superoxide dismutase; GSNO, *S*-nitrosoglutathione; SNOC, *S*-nitrosocysteine; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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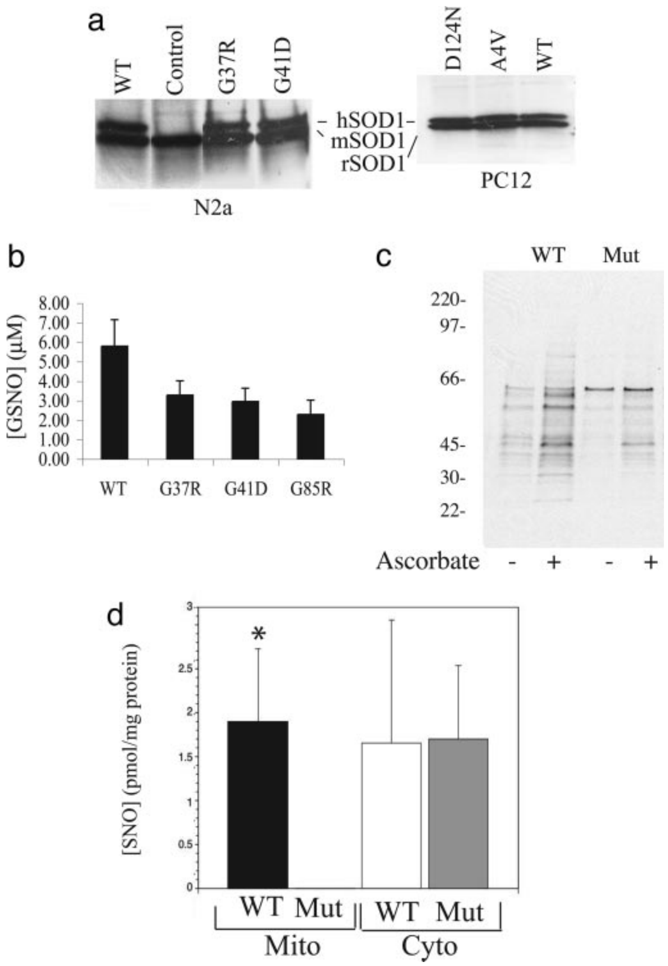

Fig. 1. Decreased peptide and protein S-nitrosylation in cell lines expressing SOD1 mutants. (*a*) SOD1 expression levels in N2a or PC12 cells stably transfected with empty control vector (Control), or vectors expressing human WT SOD1 (WT), or ALS-associated SOD1 mutants (G37R, G41D, D124N, or A4V). Bands corresponding to human SOD1 (hSOD1) and endogenous mouse (mSOD1) or rat SOD1 (rSOD1) are shown. (*b*) GSNO levels in cell lysates obtained from N2a cells expressing either WT or mutant (G37R, G41D, G85R) SOD1 were measured by LC-MS. The data represent the mean ± SEM of four to six independent experiments. The pooled GSNO values in cells expressing SOD1 mutants are significantly lower than the GSNO values in cells expressing WT SOD1 ($P = 0.01$, unpaired two-tailed *t* test). (*c*) Whole-cell lysates from PC12 cells expressing WT or D124N mutant (Mut) SOD1 were treated with 1 μM GSNO for 1 h at room temperature in the dark. Protein S-nitrosylation in the lysates was then determined by using a biotin switch assay. Biotin labeling that increases in the presence of ascorbate (+ascorbate) is indicative of protein S-nitrosylation. The gel is representative of three comparable experiments. (*d*) Total SNOs in mitochondrial (Mito) and cytoplasmic (Cyto) lysates of N2a cells expressing WT or mutant (Mut) SOD1 (G37R and G41D) were measured by chemiluminescence. *, $P = 0.03$ versus mutant mitochondria, paired *t* test, $n = 8$.

with WT SOD1 (13). Smaller increases in denitrosylase activity are seen when comparing mutant versus WT SOD1 immunoprecipitated from the spinal cords of transgenic mice (see Fig. 5, which is published as supporting information on the PNAS web site). To determine whether the increased denitrosylase activity of ALS-associated SOD1 mutants leads to an aberrant decrease in intracellular S-nitrosylation, SNO levels were measured in two different neuronal cell lines (N2a and PC12) stably expressing comparable levels of either WT SOD1 or one of several SOD1 mutants (G37R, G41D, G85R, A4V, or D124N) (Fig. 1*a*). We began by analyzing intracellular levels of GSNO, one of the most abundant S-nitrosylated peptides in the central nervous system (CNS) (14), by liquid chromatography/mass spectrometry in N2a cells. Cells expressing the SOD1 mutants G37R, G41D, and G85R had significantly lower GSNO levels as compared with cells expressing WT SOD1 (Fig. 1*b*). The GSNO levels in cells expressing WT SOD1 were very similar to the levels previously reported in rat cerebellum (14). These results suggest that the increased denitrosylase activity of SOD1 mutants leads to an aberrant depletion of GSNO levels intracellularly.

GSNO-Induced Protein S-Nitrosylation Is Decreased in Cells Expressing SOD1 Mutants. GSNO serves as a reservoir of NO bioactivity in cells and transfers its NO group to proteins in transnitrosation reactions (15). To determine whether not only GSNO levels but also GSNO-induced protein S-nitrosylation are decreased in cells expressing SOD1 mutants, lysates of PC12 cells expressing either WT or mutant (D124N) SOD1 were treated with 1 μM GSNO. GSNO-induced protein S-nitrosylation was analyzed by using a biotin switch assay in which S-nitrosylated proteins are selectively labeled with biotin and then detected on an anti-biotin immunoblot. Biotin labeling that increases after ascorbate treatment is indicative of protein S-nitrosylation (16). GSNO-induced protein S-nitrosylation was decreased in the lysates of cells expressing mutant SOD1 as compared with WT SOD1 (Fig. 1*c*). Similar results were obtained by using N2a cells expressing G37R mutant SOD1 as compared with controls (data not shown). These data suggest that not only GSNO levels but also transnitrosation of proteins by GSNO are aberrantly decreased in cells expressing SOD1 mutants.

SNO Levels Are Decreased in the Mitochondria of Cells Expressing SOD1 Mutants. Mitochondria may be a key site of intracellular protein S-nitrosylation (17, 18). In addition, mutant SOD1 aggregates in mitochondria, and disruption of mitochondrial function may play a pivotal role in ALS pathogenesis (19–21). To determine whether S-nitrosylated peptide and protein levels are selectively decreased in the mitochondria of cells expressing SOD1 mutants, SNO levels were measured in mitochondrial and cytoplasmic lysates of N2a cells expressing mutant or WT SOD1. Endogenous levels of S-nitrosylated proteins in N2a cells were below the limits of detection of the biotin switch assay but were detectable by using a chemiluminescence method of SNO detection (22). Low but comparable levels of endogenous SNOs were detected in the cytoplasmic lysates of cells expressing WT and mutant (G41D and G37R) SOD1 (Fig. 1*d*). Low levels of SNOs were also detected in the mitochondrial lysates of cells expressing WT SOD1. However, SNOs were undetectable in the mitochondrial lysates of cells expressing SOD1 mutants (Fig. 1*d*). These data suggest that SOD1 mutants selectively deplete SNOs in mitochondria and thereby may contribute to mitochondrial dysfunction in ALS.

SNO Levels Are Decreased in the Spinal Cords of Mutant SOD1 Transgenic Mice. To determine whether our findings in cell lines could be extended to an animal model of ALS, SNO levels were measured in transgenic mice expressing ALS-associated SOD1 mutants. Mutant SOD1 transgenic mice develop motor neuron degeneration very similar to patients with ALS and represent the best available animal model of the disease (23). S-nitrosylated protein levels were measured by the biotin switch assay in spinal cord samples obtained from G93A and G85R mutant SOD1 transgenic mice or from age-matched WT transgenic (Fig. 2*a*) or nontransgenic controls (data not shown). Protein S-nitrosylation was detected in only 33% of the spinal cord samples from G93A mice and 67% of spinal cord samples obtained from G85R mice but in 100% of spinal cord samples obtained from age-matched WT SOD1 transgenic mice or nontransgenic controls. The decrease in protein S-nitrosylation in the mutant SOD1 transgenic mice was seen at all stages of disease, including pre-disease onset, at the onset of muscle weakness, and at the time of

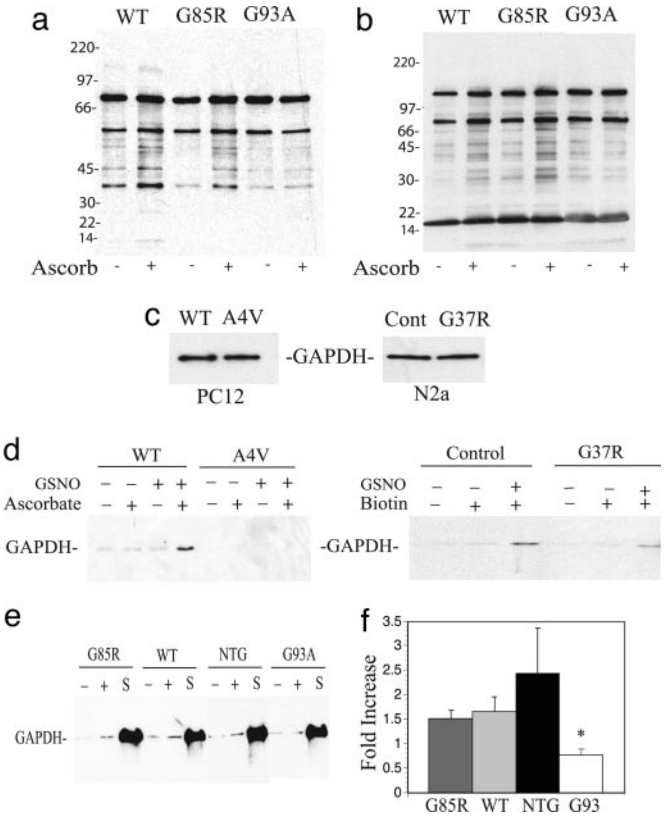

Fig. 2. Total protein and GAPDH S-nitrosylation are decreased in cell lines and the spinal cords of transgenic mice expressing SOD1 mutants. (*a*) Total protein S-nitrosylation in spinal cord lysates obtained from G85R and G93A mutant SOD1 transgenic mice at the time of paralysis or from age-matched WT SOD1 transgenic mice was assessed by the biotin switch method. Increased biotin labeling after ascorbate treatment of samples (+ascorbate) is indicative of protein S-nitrosylation. The data are representative of six separate experiments. (*b*) The experiment described in *a* was repeated using mitochondrial lysates obtained from the spinal cords of G85R and G93A mice pre-disease onset and from age-matched WT transgenic control mice. The data are representative of two separate experiments. (*c*) Levels of GAPDH in whole-cell lysates of PC12 cells expressing WT or A4V mutant SOD1, or N2a cells expressing control vector (Cont) or G37R mutant SOD1 were determined by GAPDH immunoblot analysis. The data are representative of three to four separate experiments for each cell line. (*d*) Lysates of PC12 cells expressing WT or A4V mutant SOD1, or N2a cells expressing control vector (Control) or G37R mutant SOD1 were treated with (+) or without (−) 40 μM GSNO for 1 h in the dark at room temperature. Protein S-nitrosylation was then assessed by using the biotin switch method. As controls, ascorbate or biotin were not added to some samples. The data are representative of three (N2a cells) or four (PC12 cells) separate experiments. (*e*) GAPDH S-nitrosylation was assessed by the biotin switch assay in spinal cords obtained from mutant (G85R or G93A) SOD1 transgenic mice at the onset of muscle weakness or from age-matched WT SOD1 transgenic (WT) or nontransgenic (NTG) control mice. Biotin labeling in the absence (−) and presence (+) of ascorbate and total levels of GAPDH in the starting lysates (S) of each sample are shown. The data are representative of four separate experiments. (*f*) Relative levels of GAPDH S-nitrosylation as obtained by densitometric analysis of biotin switch assays described in *e*. The data represent the mean ± SEM of ascorbate-induced increased biotin labeling of GAPDH over background biotin labeling from three to five separate experiments. *, $P = 0.016$ versus WT, 2-tailed *t* test for independent samples.

paralysis. Immunostaining of spinal cord sections with anti-SNO antiserum suggests that SNOs may be depleted in both neurons and nonneuronal cells in the spinal cords of mutant SOD1 transgenic mice (see Fig. 6, which is published as supporting information on the PNAS web site). Thus, mutant SOD1 expression aberrantly lowers protein S-nitrosylation not only in cell lines but also in the spinal cord of transgenic mice.

SNO Levels Are Higher in Spinal Cord Mitochondria of Transgenic Mice Expressing a Copper-Deficient as Compared with a Copper-Replete SOD1 Mutant. Protein S-nitrosylation was higher in the spinal cords of G85R transgenic mice as compared with G93A transgenics, and in some cases was equivalent to SNO levels in age-matched WT transgenics (Fig. 2*a*). It is possible that SNO levels are higher in G85R transgenics because the G85R mutant binds very little copper (24), and copper may be required for SOD1-induced SNO catabolism. Moreover, SOD1 mutants that bind very little copper, such as G85R, do not develop mitochondrial vacuoles and in some instances have slower disease progression than transgenics expressing copper-replete SOD1 mutants (25–27). These data raise the possibility that mitochondrial vacuolization occurs only in cells that express SOD1 mutants that bind sufficient levels of copper to critically deplete mitochondrial SNO levels.

To test this hypothesis, mitochondrial SNO levels were measured by the biotin switch assay in spinal cords of G85R (copper-deficient) or G93A (copper-replete) mutant SOD1 transgenic mice or from the spinal cords of age-matched WT SOD1 transgenic controls. Even pre-disease onset, mitochondrial SNOs were below the limits of detection in the spinal cords of G93A mutant SOD1 transgenic mice (Fig. 2*b*). In contrast, mitochondrial SNO levels in G85R mice were comparable to the levels seen in WT transgenic controls (Fig. 2*b*). These findings support the hypothesis that mitochondrial vacuolization develops only in transgenic mice expressing SOD1 mutants that bind sufficient copper to deplete mitochondrial SNO levels.

GSNO-Induced GAPDH S-Nitrosylation Is Decreased in Cells Expressing SOD1 Mutants. We next investigated the specific proteins that have aberrantly decreased S-nitrosylation in cells expressing SOD1 mutants. We began by analyzing S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) because GAPDH is known to be S-nitrosylated in neurons (16). Total levels of intracellular GAPDH were comparable in cell lines expressing mutant versus WT SOD1 (Fig. 2*c*). Although endogenous GAPDH S-nitrosylation was below the limits of detection of the biotin switch assay in both PC12 and N2a cell lines, GAPDH S-nitrosylation was detectable after lysates were treated with GSNO (40 μM) (Fig. 2*d*). GSNO-induced GAPDH S-nitrosylation was significantly decreased in cells expressing mutant as compared with WT SOD1 (Fig. 2*d*).

GAPDH S-Nitrosylation Is Decreased in the Spinal Cords of Transgenic Mice Expressing SOD1 Mutants. To determine whether GAPDH S-nitrosylation is also decreased in the spinal cord of transgenic mice expressing SOD1 mutants, biotin switch assays were performed on spinal cords obtained from mutant SOD1 transgenic mice at the onset of muscle weakness and from age-matched WT and nontransgenic control mice. Unlike the N2a and PC12 cell lines, the spinal cords had sufficiently high levels of endogenous GAPDH S-nitrosylation to be detectable by the biotin switch assay. Endogenous GAPDH S-nitrosylation was significantly reduced in three of three spinal cords of G93A transgenic mice as compared with WT and nontransgenic controls (Fig. 2 *e* and *f*). GAPDH S-nitrosylation was not clearly decreased in the spinal cords of G85R mice, again suggesting that reductions in SNO levels are milder in cells expressing the copper-deficient G85R mutant as compared with cells expressing SOD1 mutants that bind normal levels of copper such as A4V and G37R (Fig. 2*d*) and G93A (Fig. 2 *e* and *f*).

Levels of GAPDH in the Nucleus and Mitochondria Are Decreased in Cells Expressing SOD1 Mutants. GAPDH was originally thought to play a role exclusively in glycolysis. However, more recent studies have demonstrated that GAPDH has multiple nonglycolytic functions that are dependent on its subcellular location. For instance, translocation of physiologic low levels of GAPDH into the nucleus

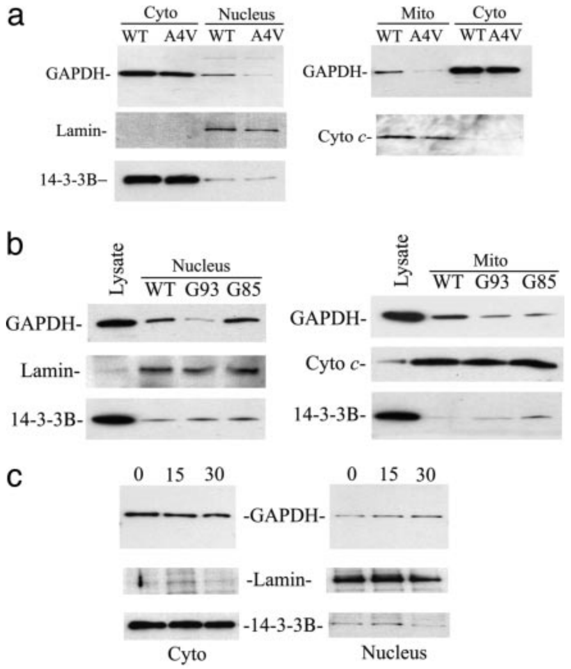

Fig. 3. Translocation of GAPDH into the nucleus and mitochondria is decreased in cells and transgenic mice expressing SOD1 mutants. (*a*) Levels of GAPDH in equal concentrations of cytoplasmic (Cyto), nuclear (Nucleus), or mitochondrial (Mito) lysates of PC12 cells expressing WT or A4V mutant SOD1 were assessed on GAPDH immunoblots. As loading controls and to assess the purity of the subcellular fractions, lamin (nucleus), 14-13-3B (cytoplasm) and cytochrome *c* (cyto *c*, mitochondria) immunoblots of the subcellular fractions were performed. The experiment is representative of three (mitochondria) or eight (nucleus) separate experiments. Similar results were obtained by using PC12 cells expressing D124N mutant SOD1. (*b*) Immunoblots of GAPDH levels in equal concentrations of nuclear (Nucleus) or mitochondrial (Mito) fractions obtained from the spinal cords of G93A (G93) or G85R (G85) mice pre-disease onset or from age-matched WT transgenic controls (WT) are shown. Lysate indicates unfractionated whole-cell WT lysate. The data are representative of two separate experiments. (*c*) PC12 cells expressing A4V SOD1 were exposed to SNOC for 0, 15, or 30 min. Levels of GAPDH in the nuclear and cytoplasmic fraction were then determined by GAPDH immunoblot analysis. The data are representative of five separate experiments.

regulates DNA repair, transcription, nuclear RNA export, and telomere maintenance (28). In contrast, translocation of higher pathophysiologic levels of GAPDH into the nucleus contributes to apoptotic cell death. Of interest, a recent report (29) demonstrated that S-nitrosylation of GAPDH regulates its transport into the nucleus during apoptosis. Specifically, S-nitrosylation of GAPDH augments its binding to Siah1, an E3-ubiquitin ligase. The nuclear localization signal of Siah1 then mediates nuclear translocation of the Siah1/GAPDH complex (29).

To determine whether decreased S-nitrosylation of GAPDH in cells expressing SOD1 mutants is associated with an aberrant decrease in GAPDH translocation to the nucleus, PC12 cells expressing mutant (A4V or D124N) or WT SOD1 were separated into nuclear and cytoplasmic fractions. The level of GAPDH in each fraction was assessed on GAPDH immunoblots. Nuclear GAPDH levels were significantly decreased in cells expressing mutant SOD1 (Fig. 3*a*). In addition, GAPDH levels were reduced in the mitochondria of mutant SOD1-expressing cells (Fig. 3*a*). Moreover, GAPDH levels were decreased in the nuclear and mitochondrial fractions of spinal cords obtained from G93A mutant SOD1 transgenic mice (Fig. 3*b*). Mitochondrial but not nuclear GAPDH levels were decreased in G85R transgenic spinal cord lysates (Fig. 3*b*), again suggesting an intermediate phenotype of G85R (Fig. 3*b*). These data raise the possibility that decreased GAPDH S-nitrosylation is associated with a disruption in nuclear and/or mitochondrial GAPDH translocation in cells and transgenic mice expressing SOD1 mutants.

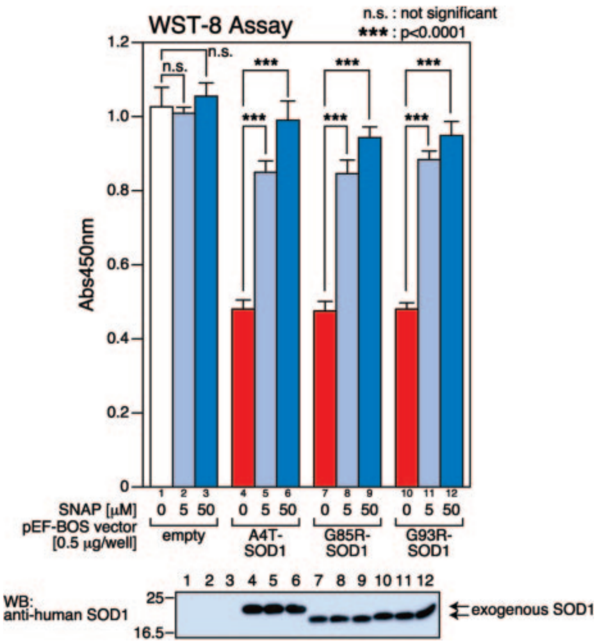

Fig. 4. Repletion of intracellular SNO levels with SNO donor compounds protects against mutant SOD1-induced toxicity. NSC34 cells were transiently transfected with empty vector (empty) or vectors expressing the SOD1 mutants A4T, G85R, or G93R. After transfection, the cells were treated with 0, 5, or 50 μM SNAP. After 72 h, cell viability was determined by the WST-8 assay, and mutant SOD1 expression was determined on immunoblots. The numbered lanes on the immunoblots correspond to samples 1–12 in the WST-8 assay. The data represent the mean ± SD, $n = 3$. Statistical analysis was performed with one-way factorial ANOVA followed by Scheffé's probable least-squares difference (PLSD). ***, $P < 0.001$.

SNO Donors Increase the Translocation of GAPDH into the Nucleus in Cells Expressing SOD1 Mutants. We next determined whether deficient GAPDH nuclear translocation in cells expressing SOD1 mutants could be corrected with SNO donor compounds. PC12 expressing the SOD1 mutant A4V were treated for various time periods with the SNO donor compound *S*-nitrosocysteine (SNOC) (25 μM). SNO donor treatment increased GAPDH translocation into the nucleus (Fig. 3*c*), suggesting that GAPDH nuclear translocation is regulated by S-nitrosylation.

Although it is well established that increased nuclear translocation of GAPDH triggers apoptotic cell death (30), it is also possible that deficient translocation of GAPDH into the nucleus contributes to motor neuron death by preventing GAPDH from performing its normal physiologic functions in the nucleus, such as telomere maintenance and DNA repair. Moreover, S-nitrosylated GAPDH may be targeted to the nucleus by means of an association with Siah1 not only in apoptotic cells but also in resting cells.

However, we were unable to confirm that Siah1/GAPDH associations were decreased in mutant SOD1-expressing cells because the levels of endogenously S-nitrosylated GAPDH in our cell lines were too low to detect GAPDH/Siah1 associations consistently in coimmunoprecipitation experiments, even in WT SOD1-expressing cells.

SNO Donor Compounds Increase the Survival of Cells Expressing SOD1 Mutants. We next determined whether replenishing intracellular SNO levels with SNO donor compounds not only corrects deficiencies in nuclear GAPDH levels but also increases the survival of cells expressing SOD1 mutants. Mutant SOD1-induced toxicity is evident after transient transfection of the motor neuron-derived cell line NSC34 with vectors expressing SOD1 mutants. Therefore, to assess the effects of SNO donor compounds on mutant SOD1-induced toxicity, NSC34 motor neuron cells were transiently transfected with vectors expressing either WT or mutant (A4T, G85R, and G93T) SOD1. Mutant SOD1 expression (Fig. 4) but not WT SOD1 expression (data not shown) led to a 50% decrease in cell survival. However, the SNO donor compounds *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Fig. 4) or SNOC (data not shown) reversed the toxic effects of SOD1 mutants. In contrast, equal concentrations of *N*-acetylpenicillamine had no significant effect on mutant SOD1 toxicity (data not shown), indicating that the NO group of SNAP is necessary for its protective effects. These data suggest that increasing intracellular SNO levels with SNO donor compounds increases the survival of cells expressing SOD1 mutants, perhaps by correcting abnormalities in cell-signaling pathways regulated by S-nitrosylation.

Discussion

Our findings support the hypothesis that the increased denitrosylase activity of SOD1 mutants contributes to motor neuron death in ALS by depleting intracellular SNOs and disrupting the function and/or subcellular localization of proteins regulated by S-nitrosylation. Mutant SOD1-induced denitrosylation is particularly prominent in mitochondria. There is increasing evidence that mitochondrial dysfunction plays an important role in the pathogenesis of ALS. Transgenic mice develop vacuolar mitochondrial degeneration 2 to 3 months before motor neuron death, raising the possibility that mutant SOD1-induced mitochondrial degeneration contributes to motor neuron death (19). Mitochondrial abnormalities have also been reported in humans with sporadic ALS (31, 32). A subpopulation of both mutant and WT SOD1 is imported into mitochondria (33, 34). Therefore it is possible that the mitochondrial subpopulation of mutant SOD1 directly damages mitochondria. Indeed, expression of mutant SOD1 in mitochondria but not in the nucleus or endoplasmic reticulum leads to cell death (35). Moreover, mutant but not WT SOD1 forms aggregates in spinal cord mitochondria that trap Bcl-2 and may thereby deplete cells of this antiapoptotic protein (20, 21). Our studies raise the possibility that depletion of mitochondrial SNOs by SOD1 mutants disrupts the function of mitochondrial proteins and thereby also contributes to mitochondrial dysfunction and motor neuron death in ALS.

Although the excessive denitrosylase activity of SOD1 mutants may play a pathophysiologic role in ALS, the lower denitrosylase activity of WT SOD1 may play an important physiologic role in SNO homeostasis. However, WT SOD1 is a relatively inefficient denitrosylase (13, 36). Therefore, WT SOD1 is likely to denitrosylate only SNOs residing in privileged sites that are inaccessible to more efficient denitrosylases such as GSNO reductase. Because GSNO reductase in not known to reside in mitochondria, WT SOD1 may play a key role in regulating mitochondrial SNO levels.

Immunostaining studies suggest that SNOs may be depleted in both neurons and nonneuronal cells in the spinal cord of mutant SOD1 transgenic mice. Interactions between mutant SOD1-expressing nonneuronal cells and motor neurons are likely to be involved in ALS pathogenesis because expression of mutant SOD1 only in astrocytes (37) or neurons (38, 39) is not sufficient to cause motor neuron degeneration in transgenic mice. Therefore, it is possible that a disruption of SNO signaling in more than one cell type contributes to motor neuron degeneration.

In addition to disrupting cell signaling, mutant SOD1-induced denitrosylation of thiols may lead to the formation of aberrant disulfides that contribute to protein aggregation in cells expressing SOD1 mutants (40). Protein inclusions are found in the anterior horn of mutant SOD1 transgenic mice and in patients with sporadic and familial ALS (41, 42). Although the role of protein aggregation in the pathogenesis of ALS remains to be determined, it is possible that aggregates disrupt cellular function and contribute to motor neuron death.

Our studies focused on the effects of familial ALS-associated SOD1 mutations on SNO levels, but they may also be of relevance to the 95% of ALS patients with a sporadic form of the disease that is not associated with SOD1 mutations. The similar pathology in sporadic and familial ALS suggests that the two forms of the disease share common pathogenic mechanisms. It is possible that SOD1-independent mechanisms of SNO depletion, such as altered levels of intracellular reductants or NOS activity, contribute to the pathogenesis of sporadic ALS.

Our results also support the concept that NOS activity is not the sole determinant of SNO levels and that SNO breakdown may result in disease (4). Moreover, in every disease to date that is linked to altered SNO levels, the culprit protein is different. For instance, preeclampsia is associated with elevated levels of S-nitrosylated albumin (43), Parkinson's disease is associated with elevated levels of S-nitrosylated parkin (1, 2), sickle cell disease is associated with decreased levels of S-nitrosylated hemoglobin (5), asthma is associated with decreased levels of GSNO (4), and here we show that ALS is associated with decreased levels of S-nitrosylated GAPDH. The emerging paradigm is that disease pathogenesis may relate to alterations in a specific class of SNO rather than to total SNO levels or NOS activity.

In summary, our findings indicate that not only increased protein S-nitrosylation (1, 3) but also decreased S-nitrosylation of specific proteins may contribute to disease pathogenesis in the brain. The results may be of particular pathophysiologic and therapeutic relevance to diseases such as Alzheimer's, Parkinson's, and prion-associated diseases in which aberrant metal catalysis may lead to SNO depletion (44). Moreover our studies raise the possibility that SNO donor compounds may be of therapeutic efficacy in ALS and other diseases associated with deficient S-nitrosylation.

Materials and Methods

Cell Lines and Reagents. Details concerning the cell lines and reagents used in these experiments are provided in *Supporting Methods*, which is published as supporting information on the PNAS web site.

Transgenic Mice. Mice transgenic for mutated human SOD1 G93A and WT human SOD1 (23) were obtained from The Jackson Laboratory. Mice transgenic for G85R were kindly provided by D. Cleveland (41). All mice were bred for >10 generations with FVB females at the University of Massachusetts Medical School animal facility. Spinal cords were obtained from G93A and G85R mutant SOD1 transgenic mice pre-disease onset, at the onset of disease, and at the time of paralysis as described (19). Spinal cords were also obtained from age-matched WT SOD1 transgenic and nontransgenic littermate controls. The human SOD1 expression levels in WT SOD1 transgenic mice are equivalent to the human SOD1 expression in the G93A transgenics and higher than the human SOD1 expression in the G85R transgenics.

Measurement of GSNO Levels in Cell Lysates by Liquid Chromatography/Mass Spectrometry (LCMS). GSNO was measured by LCMS as described (14) with minor modifications. Details are provided in *Supporting Methods*.

SOD Transfection of Tet-on PC12 Cells. Details concerning the SOD transfection of PC12 cells expressing the reverse tetracycline-controlled transactivator (Tet-on) are provided in *Supporting Methods*.

Biotin Switch Assay. Biotin switch assays of S-nitrosylated proteins in cell lines and spinal cords were performed as described (16, 45). Details are provided in *Supporting Methods*.

Preparation of Nuclear Lysates. Nuclear lysates were prepared by using the CelLytic NuCLEAR Extraction Kit (Sigma). Details are provided in *Supporting Methods*.

Preparation of Mitochondrial Lysates. Cells (60×10^6) were washed in PBS and then resuspended in 1 ml of buffer A (250 mM sucrose/5 mM Tris, pH 7.5/50 mM KCl/1 mM EDTA/2 mM $MgCl_2$/protease inhibitors). The cells were then homogenized by using a ball bearing homogenizer (H and Y Enterprise, Redwood City, CA) with a 0.1564-inch bore size and a 0.1558-inch ball bearing until >80% of the cells stained with trypan blue. Mitochondrial and cytoplasmic fractions were then obtained by differential centrifugation as described (46).

Immunoblotting of Transfected Cell Lines. Whole cell, cytoplasmic, nuclear, or mitochondrial lysates (2.5–50 μg per lane) were separated by standard SDS/PAGE and then transferred onto nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes. Membranes were blocked with blocking buffer [PBS containing 5% milk and 20 mM Tris (pH 8.8) or PBS-Tween containing 10% milk], and then were incubated with primary antibody followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia, Bio-Rad, and Santa Cruz Biotechnology). Immunoreactive bands visualized by ECL (Amersham Pharmacia Biotech).

Treatment of Cell Lines with SNOC. SNOC was prepared fresh by combining equal volumes of 600 mM $NaNO_2$ in H_2O with 600 mM L-cysteine in 0.5 M HCl to yield a 300 mM stock solution of SNOC. PC12 cells (10×10^6) expressing A4V SOD1 and growing at log phase were treated with 25 μM SNOC for 0, 15, or 30 min. After this treatment, nuclear and cytoplasmic fractions were prepared and analyzed on GAPDH immunoblots.

Analysis of SNO Levels in Mitochondrial and Cytoplasmic Lysates by Chemiluminescence. Forty microliters of mitochondrial or cytoplasmic lysates from N2a cells expressing WT or mutant SOD1 were incubated in the presence or absence of 2.2 mM $HgCl_2$ for 10 min at room temperature and 20 min on ice in the dark. The samples were then injected into the reaction chamber of a Nitric Oxide Analyzer (Sievers, Boulder, CO) containing 50 mg of KI diluted in 1 ml of H_2O and 5 ml of glacial acetic acid. The increase in NO signal above background in the $HgCl_2$-treated samples was the SNO-specific signal. Nitrite and GSNO standards were used to quantify the amount of NO released. The limit of detection of this assay is ≈10 nM SNO. The protein concentration in each sample was determined by using a MicroBCA kit according to the manufacturer's instructions (Pierce).

Transient Transfection, SNOC, SNAP, or NAP Treatment, and Cell Viability Assay. NSC34 cells (47) (7×10^4 cells per well in a six-well plate), cultured in DMEM plus 10% FBS for 12–16 h, were transfected with pEF-BOS vectors encoding familial ALS-linked SOD1 mutants (A4T, G85R, and G93R) by the lipofection method [SOD1 vectors, 0.5 mg; LipofectAMINE (Invitrogen), 1 ml; PLUS Reagent (Invitrogen), 2 ml per well] in the absence of serum for 3 h. Cells were then incubated with DMEM plus 10% FBS for 21 h. At 24 h after the onset of transfection, the medium was changed to DMEM plus N2 supplement (GIBCO/BRL). At 72 h after the onset of transfection, cell viability was measured by the WST-8 assay with a spectrofluorimeter (Wallac1420 ARVOsx Multi Label Counter), as described (48). WST-8 assay was performed with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt, by using the Cell Counting kit-8 (Wako Pure Chemical, Osaka). Indicated concentrations (5 or 50 μM) of SNAP or freshly prepared SNOC were added at 3, 12, 24, 36, 48, and 60 h after the onset of transfection.

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